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One of the most interesting challenges facing paleobiologists is explaining the Cambrian explosion, the dramatic appearance of most metazoan animal phyla in the Early Cambrian, and the subsequent stability of these body plans over the ensuing 530 million years. We propose that because phenotypic variation decreases through geologic time, because microRNAs (miRNAs) increase genic precision, by turning an imprecise number of mRNA transcripts into a more precise number of protein molecules, and because miRNAs are continuously being added to metazoan genomes through geologic time, miRNAs might be instrumental in the canalization of development. Further, miRNAs ultimately allow for natural selection to elaborate morphological complexity, because by reducing gene expression variability, miRNAs increase heritability, allowing selection to change characters more effectively. Hence, miRNAs might play an important role in shaping metazoan macroevolution, and might be part of the solution to the Cambrian conundrum.

Keywords: body plan; evolution; metazoa; microRNAs

Introduction

This is a very exciting time to be a paleontologist. Having come from being the stratigrapher's hand maiden, and eliciting comments like Sir Peter Medawar's deprecatory statement that 'palaeontology is a particularly undemanding branch of science',[1] paleontology has now become an intellectual force all its own, attacking deep and profound problems in evolutionary theory,[2] and making major strides in our understanding of the history of life.[3–7] A recent development in paleontology is the coupling of the genetic with the geologic fossil records, a discipline called molecular paleobiology.[8] Molecular paleobiology has its origins in Bruce Runnegar's vision that paleontologists should become fluent in reading both of life's historical records, the geologic *and* the genetic,[9] and since Runnegar's pioneering agenda was proposed, paleontologists have supplemented their understanding of the geologic record with molecular data to test numerous hypotheses, ranging from the notion of hierarchical selection[10,11] to the genomic origins of biomineralization.[12] But we see at least a small part of paleontology's future being the unraveling of the molecular basis underlying the geologically rapid appearance of animal body plans in the Early Cambrian, and the mechanistic basis for the very notion of animal body plans themselves.[13]

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The Cambrian conundrum

Beginning some 555 million years ago the Earth's biota changed in profound and fundamental ways, going from an essentially static system billions of years in existence[14,15] to the one we find today, a dynamic and awesomely complex system whose origin seems to defy explanation. Part of the intrigue with the Cambrian explosion is that numerous animal phyla with very distinct body plans arrive on the scene in a geological blink of the eye, with little or no warning of what is to come in rocks that predate this interval of time. The abruptness of the transition between the ''Precambrian'' and the Cambrian was apparent right at the outset of our science with the publication of Murchison's *The Silurian System*, a treatise that paradoxically set forth the research agenda for numerous paleontologists – in addition to serving as perennial fodder for creationists. The reasoning is simple – as explained on an intelligent-design t-shirt.

Fact: Forty phyla of complex animals suddenly appear in the fossils record, no forerunners, no transitional forms leading to them ''a major mystery,'' a ''challenge.'' The *Theory* of Evolution – exploded again (idofcourse.com).

Although we would dispute the numbers, and aside from the last line, there is not much here that we would disagree with. Indeed, many of Darwin's contemporaries shared these sentiments, and we assume – if Victorian fashion dictated – that they would have worn this same t-shirt with pride. Darwin[16] writes (pp. 306–307):

> *On the sudden appearance of groups of allied species in the lowest known fossiliferous strata.* – There is another

> and allied difficulty, which is much graver. I allude to the manner in which numbers of species of the same group, suddenly appear in the lowest known fossiliferous rocks. . .
>
> . . .Several of the most eminent geologists, with Sir R. Murchison at their head, are convinced that we see in the organic remains of the lowest Silurian [sic. Cambrian] the dawn of life on this planet.

Darwin's[16] explanation for the Cambrian explosion was that the fossil record was incomplete, but since Darwin penned his hypothesis over 150 years ago, we have learned two immutable facts about the late Precambrian fossil record. First, although chock full of organic forms, the Ediacaran is remarkably reticent with its animal ancestors—besides sponges[17–19] only *Kimberella* has received broad acceptance as a metazoan, possibly a molluscan metazoan.[20] And second, the geologic fossil record is a fairly accurate representation of biotic evolution such that both molecular clock analyses and paleoecological considerations agree that mobile macrophagous animals are no older than about the Ediacaran itself.[14,15,21] Thus, elucidating the materialistic basis of the Cambrian explosion has become more elusive, not less, the more we know about the event itself, and cannot be explained away by coupling extinction of intermediates with long stretches of geologic time, despite the contrary claims of some modern neo-Darwinists.[22] Indeed, as emphasized by Erwin and Davidson,[23] early morphological disparity and the temporal asymmetry of morphological innovations are known features of the fossil record,[24,25] and cannot be sidestepped with such quaint, but ultimately antiquated, neo-Darwinian prejudices. Instead, we must attack this robust pattern with fresh ideas and new data. We propose that three discoveries made over the last few years might explain, at least in part, the Cambrian conundrum, and as such defines a research agenda that will open up whole new vistas into deep-time metazoan macroevolution.

The discoveries

Starting in the early 1990s two hypotheses have been proposed to explain the Cambrian explosion, the genomic hypothesis and the empty ecospace hypothesis.[26] The genome hypothesis[27] suggests that the metazoan genome has changed through time, initially allowing for a relatively broad exploration of metazoan morphospace, but becoming more and more canalized since the Cambrian, which generally precluded the ability to evolve new high-level morphological innovations once phyla evolved. Alternatively, the empty ecospace hypothesis[28,29] suggests that rather than a temporal asymmetry of morphological innovations, a temporal asymmetry of the *success* of these origins occurred, in which high-level morphological innovations become harder and harder through time to establish in the marine biosphere.[24,26,30] In other words, this ecological preclusion model[30] predicts that morphological innovation has occurred at both the same rate and magnitude throughout the Phanerozoic, whereas the genomic hypothesis instead predicts that the ability to evolve morphological innovations decreased in both rate and magnitude through geologic time.

The mechanistic basis of the Cambrian explosion is probably not an either-or proposition that requires deciding between genomic *versus* ecospace arguments. Rather, it requires workers to tease apart the roles each of these domains might have played in the early animal evolution. An ecological component to the Cambrian explosion is inescapable. The marine ecosystem of the Ediacaran was primarily benthic, with macroscopic organisms largely restricted to the sediment–water interface, whereas the explosion of animals in the Cambrian changed this two-dimensional world into one of three dimensions with macrophagous eumetaozans invading both the infaunal benthos as well as the pelagos.[21,31] In fact, the origin of these macrophagous mobile metazoans early in the Ediacaran is most likely the trigger of the Cambrian explosion itself.[14,15,21,31]

But, of course, these niches could not be exploited until phenotypes that could exploit them could evolve, and thus there must be a genomic component to the Cambrian explosion as well.[32,33] Nonetheless, two problems exist when thinking about this genomic hypothesis. First, contrary to expectation,[34,35] the genomes of protostomes and deuterostomes, the animals that make up the taxonomic bulk of the ''Cambrian explosion,'' are not only similar in terms of the developmental tool kit (*i.e.*, the types and diversity of components that regulate gene expression), but much of this tool kit is now known to exist in cnidarians and even sponges.[8] Second, when thinking about the subsequent constraints upon phylum-level body plan evolution, if genomic constraints are operational in metazoan macroevolution, then they must have been acquired numerous times independently by each major phylum of animals.[30] However, since 2001, three discoveries have been made that impinge greatly upon our understanding of the Cambrian explosion and the temporal asymmetry of morphological innovation, showing that not only was morphological variation higher in earlier representatives as compared to later representatives, but that protostomes and deuterostomes have indeed acquired numerous and novel genes with each phylum having its own unique repertoire, and that these genes are continually being acquired by animals through geologic time. We hypothesize that these genes, known as microRNAs (miRNAs), serve to both increase complexity and canalization, and thus they might shape, at least in part, the macroevolutionary history of Metazoa.

Discovery 1: morphological variation in trilobites was higher in earlier representatives as compared to later representatives

Both the ecospace and genomic hypotheses require that morphological variation was higher in early representatives as opposed to later – the former because the newly established ecology was still largely devoid of many niches;[36] the latter because of the nature of the newly established gene regulatory networks governing phenotype itself. However, beyond largely anecdotal evidence,[26] there was no proof that early representatives differed from latter representatives in terms of their capacity for phenotypic plasticity. With the publication of Webster's beautiful study on the decline of polymorphisms (*i.e.*, multiple character states) in trilobite morphology through their evolutionary history,[37] we can finally say with some degree of certainty that at least one group of metazoans was more variable early in their evolutionary history than later. Webster showed that earlier and/or phylogenetically more basal taxa have a higher level of intraspecific polymorphisms, and hence a higher level of phenotypic variance, as compared to younger and/or more derived taxa (Fig. 1), quantifying what had long been suspected of not only trilobites,[38] but of Cambrian taxa in general.[39,40]

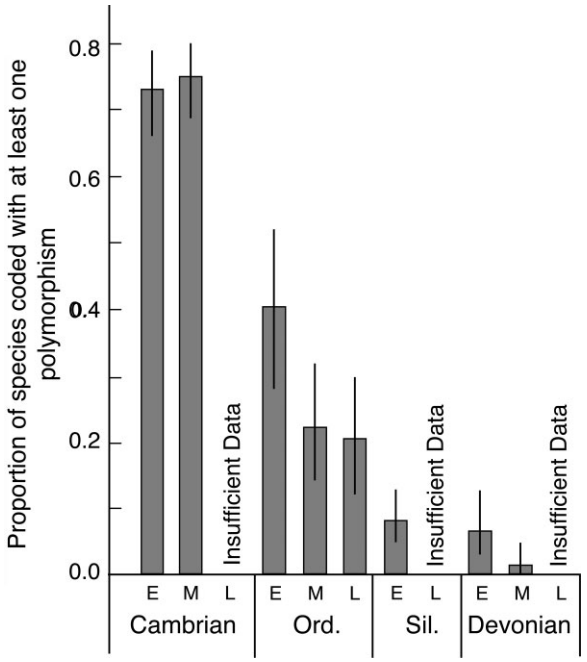

Figure 1. Webster's[37] quantification that polymorphisms, and hence phenotypic plasticity, decreases through geologic time. Shown is the temporal pattern of relative proportion of trilobite species coded as polymorphic in at least one character. Insufficient data reflect time bins where less than 40 species were available for analysis.[37] Redrawn from Webster.[37]

Webster's study fits nicely with our understanding of disparity as a whole in that a taxon's exploration of morphospace is usually achieved early in the evolutionary history of that clade.[25,41] This observation is in stark contrast to the predictions of neo-Darwinians who hypothesize that disparity is the result of the extinction of intermediates, and thus should increase through geologic time. But this is patently not the case for most taxa. Indeed, as emphasized by Erwin[25] and others,[42] when the disparity of a single deposit of arthropods 505 million years in age at least equals the total morphospace achieved by recent arthropods[43] then extinction of intermediates simply cannot be a sufficient explanation for arthropod body plan disparity, especially when the origin of Arthropoda is no older than about 575 million years.[21] In other words, in this single lineage at least as much morphological diversity was achieved in the first 70 million years than has been achieved in the subsequent 505 million years. Thus, the fact that variation was higher early in a clade's history – as opposed to later – and that disparity was achieved early – and again as opposed to later – might be two sides of the same coin.

Importantly though there is a third phenomenon related to these two, namely the temporally asymmetical origins of higher Linnaean taxa. In contrast to lower Linnaean levels, such as families and genera, which continuously arise through geologic time, most skeletonized phyla and classes make their first appearance in the early Paleozoic.[29] Indeed, using first appearances of higher Linnaean ranks has long served as a proxy for measuring disparity, and although not a replacement for true quantitative analyses, the insights gleaned from this approach have largely been confirmed by these quantitative studies.[25] Therefore, we find confirmation within Webster's study of the idea that early in a clade's history, characters vary in ways not seen since,[44] which could allow for the rapid and non-random exploration of morphospace and lead to the generation of relatively high Linnaean ranks when these taxa are classified by systematists.

Discovery 2: microRNAs reduce genetic noise by decreasing genic variation in expression

As explained above, both the genomic and the ecological hypotheses predicted that characters would be more variable in earlier representatives of a clade as opposed to latter representatives. However, what was never really explicated is why the gene regulatory networks governing phenotypic output would be more ''sloppy'' in these earlier representatives[38] – what is it about the design of a network that would allow for early – but not later – exploration of morphospace. Although theoretical considerations suggested that increases to the complexity of a network would result in canalization as more and more connections increase the robustness of the

network,[45] where "robustness" refers to the invariance of the resulting phenotype in the face of perturbation,[45] it appeared to be little more than an assertion that the gene regulatory networks governing development were in some way different in animals living in the Cambrian *versus* the Cretaceous.

Recently, a new level and mode of gene regulation has been revealed that has particular relevance to this problem – negative gene regulation via miRNAs.[46–52] miRNAs are small ~22 nucleotide RNA molecules that negatively control the translation of messenger RNA molecules, either by promoting the degradation of mRNA they are bound to, and/or by preventing the translation of mRNA in a manner that is still being elucidated.[53,54] Like transcription factors, miRNAs consist of many independently derived groups or families of *trans*-acting genes that recognize a sequence-specific *cis* motif.[55] Nonetheless, a major and important distinction between miRNAs and transcription factors is in their mode of action.[49] Transcription factors, like Hox proteins or Fox proteins, recognize specific sequences in the regulatory regions of downstream target genes, usually motifs present in the 5′ or "upstream" region of the gene, and when bound they regulate the transcription of the target gene. miRNAs, on the other hand, are regulatory RNA molecules that recognize specific sequences in the 3′ untranslated region (3′UTR) of messenger RNA molecules, and once bound to a target site ultimately prevent the translation of the messenger RNA.[53]

miRNAs are part of gene regulatory networks, and depending how the miRNA is wired into the network, can have different affects on the network's output.[45] An miRNA can be wired into a "coherent feed-forward loop" whereby an miRNA is induced by a transcription factor that also represses the miRNA target genes – thus both the transcription *and* the translation of a particular target gene is down-regulated (Fig. 2a). Coherent feed-forward loop then ensures that gene products that should not be expressed in the cell at that particular point in space and time are indeed not present. Alternatively, the miRNAs can be part of what is called an "incoherent feed-forward loop," whereby both the miRNA and the target genes are induced by the transcription factor, but the miRNA negatively regulates the target gene, allowing for the "fine-tuning" of the expression level of the target gene (Fig. 2b). Importantly, miRNAs confer robustness to the network whether the network is coherent or incoherent – either by preventing ectopic protein molecules from appearing inappropriately or by buffering fluctuations in expression levels.[45] In either case, the influence of the miRNA is to reduce the amount of noise inherent in the system precisely by regulating the numbers of protein molecules produced from an imprecise number of transcripts.[56,57] Indeed, theoretical considerations suggested that one way for the cell to minimize biological noise was to maximize transcription but minimize translation per miRNA,[56,58] exactly the role miRNAs seem to play in gene regulatory networks.[59]

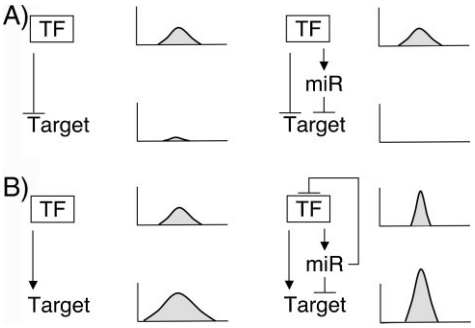

Figure 2. The role miRNAs play in buffering developmental noise. **A**: In a coherent feed-forward network a transcription factor negatively regulates a downstream target, keeping it off in a given spatio-temporal context. However, because transcription can be leaky, a few unwanted protein molecules will be expressed (left). If, however, an miRNA is added to the system (right), there is now the opportunity for both transcriptional *and* translational down-regulation, keeping spurious protein molecules from appearing that could potentially disrupt the developmental trajectory of a cell. **B**: In an incoherent feed-forward loop the transcription factor positively regulates a downstream target and an miRNA, which negatively regulates the same target (right). The affect of this will be to increase the precision of not only the transcription factor, if the miRNA feeds back into the transcription factor, but also the target genes (compare the right side with the left), given that the miRNA will reduce both the amount of transmitted noise and the amount of intrinsic noise.[56]

Because miRNAs affect the number of messenger RNA molecules, the role miRNAs potentially play in reducing noise can be ascertained by comparative microarray analysis. Wang and coworkers[60] showed that cross-species variation of messenger RNA expression levels was significantly lower if these genes had regulating miRNAs, as opposed to those not regulated by miRNAs. Further, they showed that the more *cis* targets the messenger RNA gene had in its 3′UTR for miRNA(s), the lower the cross-species variation of messenger RNA molecules themselves. Thus, precision in genic output, as measured by the variation in number of messenger RNA molecules, is achieved by miRNAs acting on messenger RNAs, not by transcription factors acting on genes.

The phenotypic consequences of affecting the amount of noise in a development system was beautifully demonstrated by the study of Li *et al.*,[61] who showed that removal of the miRNA gene *miR-9a* resulted in flies that were viable and fertile, but had a much more variable number of sense organs.[62] These sense organs are derived from a sensory organ precursor (SOP) cell that is specified, in part, by the action of the transcription factor "Senseless." Senseless protein increases in the SOP cell, and is prevented from doing so by the action of *miR-9* acting on the 3′UTR of Senseless, lowering the levels of Senseless protein in the non-SOP cells.[61,62] Because *miR-9a* sets a threshold that Senseless expression must overcome in order to trigger the requisite gene regulatory network underlying sensory organ development,[62] abrogation of *miR-9* results in the stochastic

appearance of SOP cells, and hence the appearance of variable numbers of sensory organs among individuals. Therefore, the removal of the miRNA in this system results in a character with no variation among individuals (two SOP cells per hemisegment in *Drosophila melanogaster*) becoming highly variable both within and between individuals.

Discovery 3: microRNAs are continuously being added to metazoan genomes through time

Although the messenger RNA developmental tool kit is largely conserved across Metazoa, miRNAs are not part of the original metazoan genic repertoire, as they appear to have evolved within metazoans at least twice, once in demosponges and once within eumetazoans[63] (Fig. 3; but see Grimson *et al.*[64] for an alternative perspective). Furthermore, unlike transcription factor families, new miRNA genes have been continually acquired in each eumetazoan lineage, often miRNA genes constituting novel miRNA families with unique seed sequences (Fig. 3; Table 1). Curiously, the only known place on the metazoan tree devoid of miRNA innovation is the lineage leading to the sponge *Amphimedon queenslandica* (Fig. 3). Grimson *et al.*[64] reported the presence of eight miRNAs in this taxon using deep sequencing of an *A. queenslandica* miRNA library, and all eight were found in the sponge *Haliclona*,[63] arguing that all eight evolved early in the *Amphimedon* lineage with no new miRNAs acquired after *Amphimedom* split from *Haliclona*.

This continuous acquisition of miRNA families in eumetazoan lineages means that more and more of the protein-coding repertoire comes under the control of miRNA gene regulation through geologic time.[65] And because each eumetazoan lineage is independently acquiring its own unique miRNAs, not only is the genome of an arthropod different from that of an echinoderm, in terms of which targets are being regulated at any one time during development, but an arthropod genome in the Ordovician was different from an arthropod genome in the Cambrian, and it will be different in the Silurian. But this increase in miRNA families in eumetazoan lineages is not metronomic as increased rates of acquisition of miRNA families correlate with dramatic increases to morphological complexity.[65,66] In the time during which nephrozoans (*i.e.*, protostomes and deuterostomes) acquired 32 novel miRNA families, cnidarians acquired only a single miRNA family; in the time during which vertebrates acquired 40 novel miRNA families, pancrustaceans, annelids, gastropod molluscs, and eleutherozoan echinoderms acquired only 5–8 novel families; and in the time during which primates acquired 84 novel miRNA families, rodents only acquired 16 novel families (Fig. 3,

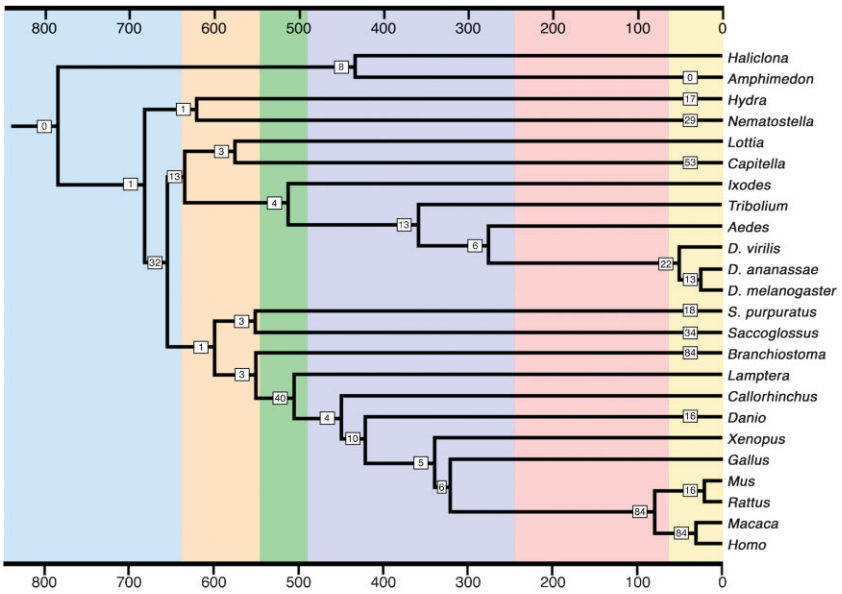

Figure 3. The acquisition of miRNA gene families from the Cryogenian (light blue), through the Cenozoic (dark yellow) for 24 metazoan taxa. miRNA family gains are shown at each node (see Table 1 for full details of the gains and losses of miRNA families for each taxonomic group considered). Note that each node is characterized by the addition of at least one new miRNA family, and all eumetazoan lineages acquire at least one novel miRNA family. Further, there are three instances of a relatively high rate of miRNA family acquisition, once at the base of the protostomes and deuterostomes, once at the base of the vertebrates, and once at the base of primates (human and macaca). The only lineage not known to have evolved new miRNAs over the last 450 million years is the demosponge *A. queenslandica*.[64] Data for the cnidarian *Hydra*, the polychaete annelid *Capitella sp.*, the sea urchin *Strongylocentrotus purpuratus*, the hemichordate *Saccoglossus kowalevskii*, and the cephalochordate (*i.e.*, amphioxus) *B. floridae* are from Peterson (unpublished); *Amphimedon* and *Nematostella* are from Grimson *et al.*[64]; all others are taken from Wheeler *et al.*[63], Sperling *et al.*[55] and miRBase v.12.

Table 1. Evolutionary acquisition of miRNA families

Taxon	miRNA family gains[a]	Inferred miRNA family losses[b]	Total number of miRNA families
Haplosclerida	8: 2014, 2015, 2016, 2017, 2018, 2019, 2020, 2021	?	8
Eumetazoa	1: (10, 99, **100**)	?	1
Cnidaria	1: 2022	0	2
Triploblastica	8: (**1**, 206), (**31**, 72), (**34**, 449), (4, 9 = **79***), (25, **92**, 363), 124, 219, (252a, 252b)	0	9
Nephrozoa	24: (**let7**, 98), 7, (**8**, 141, 200, 236, 429), (**22**, 745, 980), (**29**, 83, 285, 746), 33, 71, (**96**, 182, 183, 263), (**125**, lin4), 133, 137, 153, 184, 190, 193, 210, (**216**, 283, 304, 747), 242, 278, 281, 315, 365, 375, 2001	0	33
Protostomia	13: (**Bantam**, 80, 81, 82), (**2**, 13), 12, 36, (**67**, 307), (76, **981**), 87, 277, (**279**, 996), 317, 750, (958, **1175**), 1993	0	46
Trochozoa	3: 1989, 1992, 1994	0	49
Annelida	7: 1987, 1995, 1996, 1997, 1998, 1999, 2000	0	56
Gastropoda	5: 1984, 1985, 1986, 1988, 1990, 1991	1: 365	53
Ecdysozoa	1: 993	1: 365	46
Arthropoda	3: 275, 276, iab4	2: 242,1993	47
Pancrustacea	1: 965	1: 2001	47
Insecta	12: 14, 282, 286, 305, 927, 929, 932, 970, 988, 989, (995, **998**), 1000	1: 153	58
Diptera	6: 11, 306, 308, 316, 957, 999	1: 750	63
Drosophila	22: (**3**, 309, 318), 5, 6, 274, 280, 284, 287, 288, 289, 314, 955, 956, 962, 963, 969, 971, 976, 987, 994, 1006, 1007, 1010	2: 36,71	81
Deuterostomia	1: (**103**, 107, 2013)	0	34
Ambulacraria	3: 2008, 2011, 2012	1: (216, 283)	36
Eleutherozoa	8: 2002, 2004, 2005, 2006, 2007, 2009, 2010, 2011	1: 315	43
Chordata	3: 129, 135, 217	3: 242, 315, 2001	34
Olfactores	3: 101, 126, 155	2: 71, 278	35
Vertebrata	37: (**15**, 16, 195, 322, 424, 457, 497), (**17**, 18, 20, 93, 106), 19, 21, 23, 24, 26, 27, 30, 122, 128, (**130**, 301), (**132**, 212), 138, 139, 140, 142, 143, 145, 146, (**148**, 152), 181, (**192**, 215) 194, 196, 199, 203, (**204**, 211), 205, 214, 218, 220, 221, 222, 338, 451, 456	1: 281	72
Gnathostomata	4: 144, 150, (**425**, 731), 454	1: 252	75
Osteichthyes	10: 187, 202, (**208**, 736), (**223**, 599), 455, 458, (**459**, 802), (**460**, 730), 489, 499	0	85
Zebrafish	16: 430, 461, 462, 722, 723, 724, 725, 726, 727, 728, 729, 732, 733, 734, 735, 737	0	101
Tetrapoda	5: (191, 637), (290, 291, 292, 293, 294, 295, **302**, 371, 372, 373, 512, 515, 516, 517, 518, 519, 520, 521, 522, 523, 524, 525), 320, 367, 383	0	90
Amniota	6: 32, 147, (297, **466**, 467, 669, 1277), 490, 551, 762 (?)	0	96
Mammalia	84: (**28**, 151, 708), (95, **421**, 545, 1264), 105, 127, 134, 136, 149, (**154**, 300, 323, 369, 376, 377, 381, 382, 409, 410, 453, 487, 494, 496, 539, 655, 656, 1185), 185, 186, (**188**, 532, 660), 197, 224, 296, 298, 299, 324, 325, 326, 328, (**329**, 495, 543), 330, 331, 335, 337, 339, 340, 342, 345, 346, 350, 361, (**362**, 500, 501, 502), 370, 374, (**378**, 422), (**379**, 380, 411, 654, 758, 1197), 384, 412, 423, 431, 432, 433, 448, 450, 452, 483, 484, 485, 486, 488, 491, 493, 503, 504, 505, (465, 470, **506**, 507, 508, 509, 510, 513, 514, 742, 743, 871, 878, 880, 881, 888, 890, 892), 511, 542, 544, 568, 582, 590, 592, 598, 615, 652, 653, 664, 665, 668, 670, 671, 675, 744, 760, 764, 770, 873, 874, 875, 876, 877, 1224	3: 456, 458, 460	177
Rodentia	16: 207, 327, 343, 344, 351, 434, 463, 471, 540, 541, 672, 673, 674, 872, 879, 883	1: 432	192

Table 1. (*Continued*)

Taxon	miRNA family gains[a]	Inferred miRNA family losses[b]	Total number of miRNA families
Primates	84: 198, 492, 498, (**548**, 570, 579, 603), 549, 550, 552, 553, 554, 556, 557, 558, 562, 563, 567, 569, 572, 573, 576, 577, 578, 580, 581, 583, 584, 586, 587, 589, 593, 597, 600, 601, 604, 605, 607, 609, 611, 612, 616, 618, 619, 624, 625, 626, 627, 628, 631, 632, 633, 636, 638, 639, 640, 642, 643, 644, 648, 649, 650, 651, 657, 661, 662, 663, 765, 767, 885, 887, 889, 891, 920, 922, 924, 933, 934, 936, 937, 938, 939, 940, 942, 944, 1225, 1226	4: 350, 670, 762, 764	257

[a]Families are designated parenthetically and are underlined; the family names are given in bold. In some cases the same gene is given at least two different names (*e.g.*, miR-22 = miR-745 = miR-980), whereas in other cases there were gene duplications generating at least two copies of the gene in an individual taxon's genome (*e.g.*, miR-10 family, miR-252 family, miR-96 family). An miRNA gene that expresses both arms of the hairpin (*i.e.*, both a mature and a star) are here considered a single family, as they together constitute a single genetic innovation.
[b]Question marks indicate that it is not possible at the moment to reconstruct losses for this node.

Table 1). Indeed, the addition of these 84 novel miRNA families represents near the totality of miRNA innovation in the lineage leading to the cephalochordate *Branchiostoma floridae* (Fig. 3), and thus primates evolved almost the same number of families as amphioxus in about a tenth of the time.

Importantly, this continuous acquisition has a hierarchical component such that earlier-evolved miRNAs are expressed at higher levels and more broadly than later-evolved miRNAs.[67] For example, in heart development, two of the miRNAs that are expressed are miR-1 and miR-208 – the former evolved at the base of triploblasts as transcripts are detected in the acoel flatworm,[63] whereas the latter is restricted to vertebrates.[66] Further, in vertebrates miR-1 is also expressed in skeletal muscle (its likely primitive locus of expression) whereas miR-208 is restricted to only the heart. With respect to heart development, not only is miR-1 much more highly expressed than miR-208,[68] the phenotype resulting from the knockout is far more severe with miR-1 than it is with miR-208. Elimination of miR-1 results in a lethal phenotype with defects to cardiac morphogenesis, electrical conduction, and cell cycle control,[69] whereas elimination of miR-208 resulted in normal mice unless the heart was put under stress and only then was a phenotype manifested.[70] Thus, unlike gene regulatory networks, which contra Davidson and Erwin[71] are not intrinsically hierarchical,[72] miRNA acquisition parallels the metazoan hierarchy.

The proposal: miRNAs, canalization, and complexity

These three patterns suggest that intraspecific phenotypic variation decreases through geologic time (Fig. 1), that miRNAs decrease the variation in gene expression (Fig. 2), and that the number of miRNAs found in the genomes of a lineage increases through geologic time (Fig. 3). Further, three instances in particular, triploblasts, vertebrates, and primates, had large increases to both morphological complexity and their rate of miRNA acquisition. Hence, we propose that miRNAs might be instrumental in canalizing development[45] such that phenotypic variation decreases through geologic time at the cost of increasing developmental precision, allowing for subsequent increases in morphological complexity.[65]

It might seem paradoxical that the same molecules potentially confer both complexity and constraint, but when one considers their mode of action coupled with their unique evolution, we believe that this paradox is removed. This is because if the main consequence of miRNA regulation is to stabilize the level of gene expression, then this could make the phenotypic traits influenced by this regulated gene much more "evolvable."[73] This is so because the ability of any phenotypic trait to evolve by natural selection depends on the heritability of that trait. In its simplest expression, the change in a quantitative phenotypic trait due to natural selection over one generation is given by

$$\Delta z = h^2 S$$

where h^2 is the narrow-sense heritability of the trait z, and S is the selection differential.[74,75] The selection differential quantifies the amount of change in the mean phenotype *within* one generation caused by differences in fitness among individuals (*i.e.*, differences in their abilities to survive and reproduce based on the values of the phenotype they possess). S is thus a metric of the strength of natural selection on the trait. Heritability quantifies the phenotypic resemblance of parents and offspring caused by the genes passed between them, and thus quantifies the ability of a population to genetically respond to a given selective pressure.

The narrow-sense heritability of a trait is defined as V_A/V_P *i.e.*, the ratio between the additive genetic variation (V_A) and the total phenotypic variance (V_P) in a phenotypic trait among a group of individuals.[74,75] The additive genetic component is that component of the phenotype that can be "predicted" by a linear regression of phenotype on the alleles that comprise the genotypes in the population. The total phenotypic

variation is composed of all genetic (additive, dominance, epistasis, and interactive effects of alleles at all loci that influence the expression of this trait) and non-genetic contributors to determining the value of the trait in question. Among the non-genetic contributors to phenotype are the developmental and environmental effects that make the value of a trait produced by a genotype unpredictable.[74,75]

By stabilizing the level of gene expression, miRNA regulation of critical genes involved in the production of a phenotypic trait would substantially decrease the unpredictability of the trait value produced, thereby decreasing V_P and thus increasing the heritability of the trait. Because the additive contribution of alleles at multiple loci is a statistical property of how those particular alleles interact to produce the phenotype, miRNA regulation would not necessarily alter V_A. Whether V_A would change depends on how the average level of protein production is changed by stabilizing gene expression levels (*i.e.*, decreasing the variance in gene expression levels). For example, if miRNA regulation decreases the variance in gene expression but does not change the average level of gene expression, V_A would be unchanged, but V_P would decrease substantially. Therefore, we predict that miRNA regulation is important in the evolution of a lineage by increasing the heritability of critical phenotypes, which will make these traits substantially more responsive to the action of natural selection.

Based on these considerations, we imagine the following general scenario for the evolution of miRNA regulation. First, imagine some polygenic phenotypic trait that confers a range of fitnesses (*i.e.*, survival and reproduction) on individuals based on the values each possesses (*i.e.*, a large value of *S*), but has low heritability because of the unpredictability of the levels of a key gene product necessary for the determination of the trait value in individuals (see Fig. 4). At this point, even though selection acts to change the phenotypic distribution in each generation, populations of this species do not evolve because of the low correspondence between the trait value expressed by individuals and the alleles they possess in their genotypes. Now imagine that mutations in an expressed RNA hairpin (*i.e.*, an expressed non-coding RNA molecule that has the requisite secondary structure and thus is processed by the miRNA-processing machinery, but does not yet have any mRNA targets and for all intents and purposes is simply transcriptional noise) produces an allele that now binds to the 3′UTR of the key gene and thus stabilizes the level of the gene product in a few individuals in the population. Initially, this will only slightly decrease the unpredictability of the trait values produced in the entire population because it is only present in a small fraction of the population. Nonetheless, when the newly functional miRNA binds to the mRNAs of the coding genes in some individuals in the population, those individuals produce substantially less phenotypic variation than those with the same genotype at the coding gene but non-functional miRNA allele.

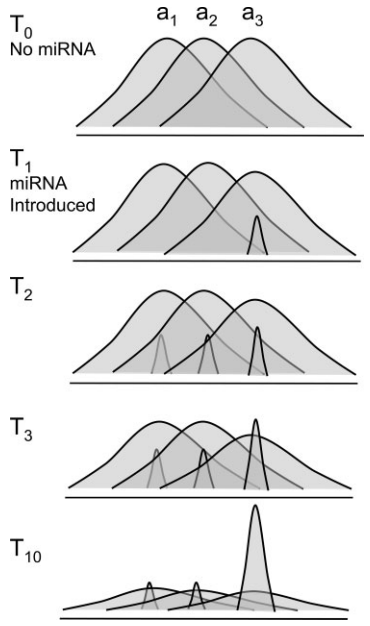

Figure 4. General scenario for the evolution of miRNA regulation. Imagine at T_0 some polygenic phenotypic trait with low correspondence between the trait value expressed by individuals and the alleles they possess in their genotypes. At T_1 mutations in an expressed RNA hairpin produces an allele that will bind to the 3′UTR of the key gene and thus stabilize the level of the gene product in an individual in the population. This mutation will spread to individuals with other alleles (T_2), but will only slightly decrease the unpredictability of the trait values produced in the entire population because it is only present in a small fraction of the population (T_2 and T_3). Natural selection can now act by favoring individuals with alleles of the coding gene conferring higher fitness and with the regulatory miRNA allele (T_3 and T_{10}).

Natural selection can now act by favoring individuals with alleles of the coding gene conferring higher fitness and with the regulatory miRNA allele. If the new functional miRNA allele increases in the population because of this selection, then over time we would expect that one allele with miRNA will go to fixation in the population (see below). Moreover, because the heritability of the trait influenced by the gene is now increased, selection can more easily move the phenotype across the fitness landscape, potentially allowing for phenotypic novelty (Fig. 5). It is important to note that in this scenario, miRNAs increase evolvability by increasing heritability, not by storing hidden genetic variability, which when released can be subject to selection as is the case in evolutionary capacitors such as Hsp90.[45,76] Further, we stress that contra assumption (*e.g.*, Liu *et al.*[77]), there would be no reason why a newly acquired miRNA would be detrimental to fitness, as there is no *a priori* reason why the conference of precision is indeed detrimental. In fact, this observation might explain, at least in part, why miRNAs, unlike novel transcription factors, can be so easily acquired through geologic time in all eumetazoan lineages thus far investigated (Fig. 3, Table 1).

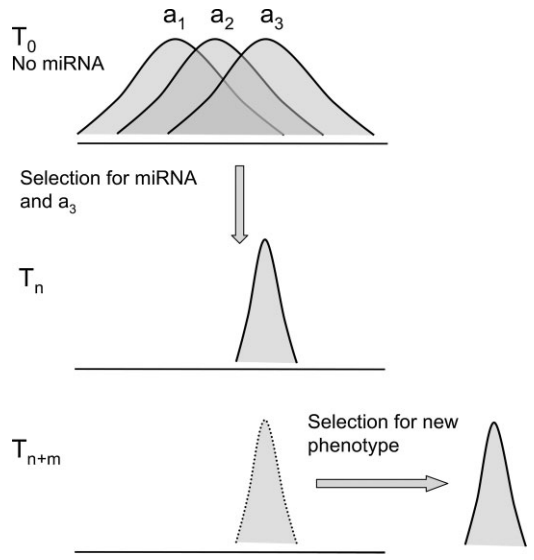

Figure 5. Two selective scenarios for an allele with miRNA regulation. First, selection of an allele (a_3) and miRNA (see Fig. 4). If the new functional miRNA allele increases in the population because of this selection, then over time we would expect that one allele with miRNA will go to fixation in the population (T_n). Second, the increased heritability of the trait influenced by the gene makes it more available to selection, which can more easily move the phenotype across the fitness landscape, potentially creating phenotypic novelty (T_{n+m}).

Exploring the proposal

To study the basics of this mechanism we built an individual-based model to simulate the evolution of the basic components of this system. An assumption of the model is that functional miRNA alleles arise from mutation(s) in a non-functional miRNA allele, which allows for the miRNA to recognize and bind a specific motif in the 3′ UTR of a coding gene. The question addressed by the simulations is whether a functional miRNA allele will increase in frequency when rare and selection acts only on the coding gene. In this model, diploid individuals had a two-locus genotype. One locus was a quantitative trait locus. To create individuals, alleles were drawn at random from a normal distribution with a mean of 0.0 and standard deviation of 1.0. These allelic values can be thought of as representing the amount of gene product produced from a coding gene. The other locus was an miRNA locus that regulated the expression of alleles at the quantitative trait locus; a non-functional miRNA allele and a functional regulatory miRNA allele segregated at this locus. An individual's phenotype was determined by adding the values of the two alleles at the quantitative trait locus.

If the individual was homozygous for the alleles coding a non-functional miRNA, another random number was drawn from a normal distribution with mean 0.0 and a specified variance and added to the phenotype to obtain the final value. This random number simulated the environmental variance inherent in the expression of a locus not regulated by an miRNA. A population of clones of one genotype would then produce phenotypes with a standard deviation equal to the specified environmental variance (T_0, Fig. 5).

If, however, the individual was either homozygous for a functional miRNA or heterozygous, no environmental variation was added to make the individual's phenotype. A population of clones of one genotype here would all have identical phenotypes (*i.e.*, no environmental variance) (*e.g.*, T_n, Fig. 5). Thus overall, populations started with an average phenotypic value of 0.0. Population size was fixed at 1000, and the initial population contained a small number of the functional regulatory miRNA allele (0.5%). Directional viability selection was applied to the population at each generation based on the phenotypic values of individuals, and the survivors of this viability selection were mated at random to start the next generation.

Simulating a number of conditions yielded the following general results. First, if the environmental variance of the phenotype for a non-functional miRNA homozygote was small, no evolution occurred at the miRNA locus, but the quantitative trait locus responded strongly to selection. Under these conditions, the miRNA locus is essentially neutral, since it has little effect on the amount of environmental variation of expression of the quantitative locus and thus the heritability of the phenotypic trait.

In contrast, if the environmental variation applied to the phenotype of the non-functional miRNA homozygote was large, two outcomes were apparent. If the functional miRNA allele was lost due to sampling in the first few iterations of a simulation, the population did not evolve, even if selection on the phenotype was strong. This is as expected because the high environmental variance made the phenotypic value of an individual unpredictable based on its genotype, *i.e.*, the heritability of the phenotype was very small. However, in many replicates, the functional miRNA rapidly increased to near fixation and concomitant with the population's phenotype rapidly increased in value. These results showed that natural selection can act indirectly at an miRNA locus to drive a functional regulatory miRNA allele to fixation because of selection acting on the locus it regulates. This indirect selection acts because the miRNA makes the expression of the phenotype produced by the quantitative trait predictable.

Implications and conclusions

These preliminary considerations suggest that if the primary function served by miRNA gene regulation is to stabilize gene expression levels, miRNA knockouts may not have strongly deleterious phenotypes in individuals,[45] as recently realized in a large-scale knockout study.[78] Although we are in very early days in terms of understanding the precise roles

miRNAs play within the context of gene regulatory networks,[79] we propose that the consequences of miRNA knockouts may be only discernable at the level of a population. This is because miRNAs do not qualitatively change the pattern of gene expression, but rather stabilize the quantitative levels of expression, and thus a knockout would only increase the variance among individuals in phenotypes that are affected by the loci under regulation. As discussed above, the removal of the *miR-9* locus in *D. melanogaster* did not affect viability or fertility, only the variance of a particular morphological character.[61] Importantly though, an miRNA knockout should decrease the heritability of a trait because of an increase in V_P as compared to lines with functional miRNA gene regulation. Consequently, selection on these traits should be ineffectual in miRNA knockout lines, but should result in strong evolutionary responses in functioning miRNA lines.

Valentine[30] stressed that the central question surrounding the genome hypothesis for the Cambrian explosion is whether Postcambrian genomes indeed acquired constraints that prevented the exploration of morphospace as compared to Precambrian and Cambrian genomes. But because the acquisition of any constraints would have to have occurred numerous times independently so that each metazoan lineage was similarly entrained, Valentine[30] suggested that the genomic hypothesis, although plausible, was an unlikely explanation of the body plan problem. But of course no one could have predicted the existence of miRNAs, the "dark matter" of the metazoan genome,[80] their continuous acquisition in all eumetazoan genomes thus far investigated, and their profound influence in regulating genic precision.

Unlike any other known component of metazoan genomes, miRNAs satisfy both of Erwin's[24] necessary conditions of relevance: 1) higher taxa should have distinctive developmental synapomorphies; and 2) unique patterns of constraint should occur within each distinctive clade. Not only can each phylum be characterized by at least one miRNA (Fig. 3), these miRNAs are totally unique with respect to miRNAs found in other phyla, meaning that they regulate different targets, and we predict confer precision in taxonomically unique ways. The discovery of miRNAs and their evolutionary dynamics has allowed us to finally be able to say with some degree of certainty that trilobite genomes *were indeed different* from echinoderm genomes, and, maybe more importantly, arthropod genomes in the Cambrian *were indeed different* from arthropod genomes in the Ordovician. Therefore, miRNAs provide both temporal and phylogenetic asymmetries, both of which are necessary for a genomic hypothesis to be a viable explanation for the origin and early evolution of animal body plans.

With the continuous addition of novel miRNAs, a greater fraction of the metazoan 'messenger RNAome' (*i.e.*, the mRNA component of the transcriptome) comes under the regulatory control of miRNAs, which we hypothesize herein confers robustness to the developmental program,[45,46] resulting in the evolution of morphological complexity[65,66] and the canalization of development through geologic time.[45] But in no way are we arguing that all of the metazoan macroevolution can be understood simply by understanding the role miRNAs play in metazoan development. Surely a large fraction of the evolutionary process is driven by *cis*-regulatory changes,[81–83] and we need to work out the details of these genetic regulatory networks if we are to understand morphogenesis and its underlying causality,[44] especially as miRNAs are newly discovered components of these gene regulatory networks. However, to focus solely on the transcription side of the equation[71] is to miss a significant part of the process. We suggest that in order to fully understand the body plan component of the Cambrian explosion, we need to understand what role the influx of numerous miRNAs had and continues to have on body plan evolution. Indeed, we foresee molecular paleobiology having much to contribute to this unique aspect of one of biology's most fascinating questions, and look forward to watching our students unravel what we think is the ultimate Gordian knot of Paleontology.

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